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PRINCIPAL INVESTIGATOR: Larry Kwak, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center
Houston, TX 77030

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Final Report (Submitted concurrently with 12-month no-cost extension request)

1. Introduction

The idea of genetic vaccination originated from the observation that injection of DNA into living animals resulted in expression of gene products *in vivo*. Preclinical studies revealed that genetic immunization induced both antigen-specific antibody and cytotoxic T lymphocyte. Early human trials of DNA vaccines have been mainly focused on infectious disease, and vaccines against malignant disease have activated anti-tumor immunity in cancer patients and extended remission. Compared with proteins or peptides, genetic vaccines are highly appealing. The antigen synthesized in host cellular machinery is naturally processed by antigen presenting cells (APC) to release all potential antigenic epitopes for priming antigen-specific immunity, avoiding HLA restriction that is absolutely required for peptide vaccine. Moreover, simplicity in large-scale production and purification makes DNA vaccines more attractive than protein. However, the goal of using DNA vaccines as a clinical therapy has not yet been achieved. The major obstacle preventing general use of DNA vaccines on patients is that the plasmid DNA, especially those encoding non-immunogenic, tumor-associated self-antigens, is relatively weak in inducing immunity. Developing an efficient strategy to enhance immunogenicity is therefore vital for translational vaccine development.

An immune adjuvant is a substance that is used to improve the efficiency of vaccine. It is usually designed to boost a critical step in the development of adaptive immunity. The downstream effects of adjuvant can be linked to enhancing the function of dendritic cells (DC) in antigen uptake and presentation. Examples include chemokines for DC recruitment, Toll-like receptor (TLR) agonists for DC maturation, and CD40 ligand and interferon- α which enhance T-cell activation. Given that development of adaptive immunity includes a series of sequential events, it can be speculated that a strategy that targets multiple steps in activation of adaptive immunity would be more beneficial than an individual adjuvant in improving the potency of vaccine.

Many adjuvant candidates such as inflammatory chemokines, TLR ligands and interferon α , are the important elements of innate immune system. Based on this concept, we hypothesized that inducing an innate immune response at vaccination sites could create an immune microenvironment containing the required elements to facilitate vaccine-triggered adaptive immunity. For example, tissue damage may present as an alternative pathway leading to innate immunity. This idea is supported by the observation that endogenous ligands of toll-like receptors are released following tissue necrosis and activate various cellular elements of the innate immune system. Hence, we reasoned that induction of controlled tissue necrosis prior to vaccination might lead to activation of innate immune system and potentiate the adaptive immune responses resulting from the vaccination. Myotoxins including cardiotoxin, especially at low doses, cause local tissue damage that is quickly resolved by muscular regeneration. When combined with a lymphoma idiotype DNA vaccine as shown in our previous report, they considerably potentiated vaccine-induced T cell immunity and tumor protection. Lymphoma idiotype is a tumor-specific antigen. The vaccine derived from lymphoma idiotype protein elicited tumor-specific immune responses and prolonged disease-free survival in patients with follicular lymphoma. Our current mechanistic study showed that cardiotoxin produced a favorable immune microenvironment at vaccination sites, triggering the recruitment and activation of antigen-presenting cells for T cell priming. Hence, we describe a novel, immune potentiating mechanism for myotoxins to improve the immunogenicity of genetic vaccines by generating an inflammatory microenvironment at vaccination sites.

2. Summary of Progress

Pre-treatment of vaccination sites with myotoxins potentiated adaptive T-cell responses to two model antigens.

We previously described a DNA vaccine which encodes lymphoma idiotype antigen (sFv) fused with monocyte chemotactic protein-3 (MCP3-sFv). This clinically relevant vaccine elicited CD8⁺ T cell-dependent protective and therapeutic anti-lymphoma immunity in mice. In our recently published study, we observed that particularly MCP3-sFv fusion DNA vaccine-induced tumor protection was significantly improved by pre-treating the vaccination sites with low dose cardiotoxin. Cardiotoxin pre-treatment was also an effective strategy to convert a less immunogenic tumor antigen into a protective tumor vaccine, as even vaccination of mice with plasmid DNA encoding a unfused sFv with cardiotoxin, but not without, protected mice from lethal tumor challenge (log rank $P=0.04$), although the level of protection was less than that induced by the fusion vaccine (Figure 1A).

Given the technical challenge of quantifying idiotype-specific CD8⁺ T-cell immunity, we used Ova model antigen to explore the mechanism that cardiotoxin acts as an immune adjuvant. Similarly, antigen-specific T-cell responses to Ova were significantly enhanced by combination of DNA vaccine encoding MCP3-Ova with cardiotoxin, compared MCP3-Ova DNA vaccine alone (Figure 1B). Overall, compared with 1.39 ± 0.28 % Ova₍₂₅₇₋₂₆₄₎ H-2K^b tetramer-positive CD8⁺ peripheral blood T cells elicited by MCP3-Ova DNA vaccination, the cardiotoxin-combined strategy elicited an average of 3.71 ± 0.5 % Ova-specific CD8⁺ T cells (the statistic data represent 3 individual experiments). Taken together, the results with these two model antigens confirm the potent, immune adjuvant effect of cardiotoxin.

Myotoxin induced sterile inflammation and DC infiltration at vaccination sites.

To elucidate the unexpected immunologic effects of cardiotoxin, we examined muscles at cardiotoxin injection sites histologically and observed marked cellular infiltration that peaked after 3 to 5 days (Figure 2A). This kinetic pattern was coincident with DNA vaccine administration on day 5. Further characterization of the infiltrated cells with cell-type specific markers revealed features of sterile inflammation, characterized by initial infiltration of granulocytes within 24 hours, followed by monocytes/macrophages on Day 3 (Figure 2B, Gr-1 and F4/80, respectively). Surprisingly, infiltrating dendritic cells (DC) were also observed by Day 3 (CD11c). Lymphocytes were not predominant (CD3 and B220).

Myotoxin-induced DC infiltration at vaccination sites was essential for the development of vaccine-induced adaptive immunity.

DC infiltration was essential for T cell immunity induced by MCP3-Ova plasmid DNA plus cardiotoxin, as depletion of DC by intramuscular injection of diphtheria toxin (DT) in DT receptor (DTR)-transgenic mice significantly reduced the number of Ova peptide₍₂₅₇₋₂₆₄₎-specific CD8⁺ T cells elicited by vaccination [Figure 3A: 0.48% for CT+DT+MCP3-Ova(DTR), vs 4.5% for CT+DT+MCP3-Ova(WT)]. Overall, MCP3-Ova+CT vaccination of DT-treated DTR mice resulted in an average of only $0.58\% \pm 0.09$ Ova tetramer positive CD8⁺ peripheral blood T cells, compared with $3.68\% \pm 0.57$ in wild type (WT) C57/BL mice ($P < 0.01$, Figure 3B). Without DT treatment, the numbers of peptide-specific T cells elicited by vaccination were comparable in DTR transgenic mice and WT mice (Figure 3B). Together, these data suggest that the induction of local sterile inflammation, including recruitment of DC, serves as the principal mechanism for cardiotoxin-enhanced DNA vaccination and induction of adaptive immunity.

Generation of sterile inflammation creates an immune microenvironment that links innate and adaptive immune mechanisms.

Cardiotoxin-induced sterile inflammation at vaccination sites likely favors both the recruitment of antigen-presenting DC and an immune microenvironment for developing adaptive immunity. Microarray analysis of total RNA isolated from cardiotoxin-injected muscle demonstrated up-regulation of select chemokines, cytokines and DC signaling molecules, which have critical roles in linking innate to adaptive immunity (Figure 4). Changes in chemokine production matched the temporal pattern of cellular infiltration (Figure 4A). For example, neutrophil-attracting CXCL1, CXCL5 and CCL9 were over-expressed on Day 1; whereas chemokines attracting monocytes and/or dendritic cells, exemplified by CCL24, CCL3, CCL22 and CXCL14, were predominantly increased after Day 3. Notable up-regulation of interferon regulatory factor (IRF) family genes was observed (Figure 4B). These cytokines have significant roles in regulating DC differentiation and function, and in integrating innate and adaptive immunity. Tumor necrosis factor superfamily factor 9 (Tnfsf9), a cytokine produced by DC, but required for T cell expansion was also up-regulated. Among DC activating signals, CD40 was substantially up-regulated (Figure 4C). Its critical role in the generation of cardiotoxin-facilitated T cell immunity was confirmed by showing that induction of peptide-specific T cells by vaccination were reduced in CD40 deficient mice (Figure 5A+B). Toll-like receptor-2 (TLR2) was up-regulated immediately after cardiotoxin injection, as well as TLR1 and TLR6, both of which can form functional dimers, as well as TLR2 endogenous ligand HMGB1 (high motility group box 1) (Figure 4C). These results are consistent with a role for DC activation, which is mediated by TLR. These findings were confirmed by the observation of impaired induction of peptide-specific CD8⁺ T cells in the peripheral blood of vaccinated MyD88 deficient mice, compared with WT C57/BL mice (Figure 5C+D). Conversely, although IL1 β receptor signaling is also mediated by MyD88, its participation in the immune potentiating effect of cardiotoxin was not required, as the number of peptide-specific T cells was not different in vaccinated IL1 β receptor deficient, compared with WT mice (Figure 5C+D). Altogether, these results suggest that the generation of sterile inflammation by cardiotoxin activates innate immunity, which in turn facilitates recruitment and activation of antigen-presenting cells and induction of antigen-specific T cell immunity by vaccination.

3. Conclusions and Future Plans

Cardiotoxin has been tested previously as an immune adjuvant for prophylactic vaccines against infectious diseases. Its principal effect is considered to be the result of increased plasmid DNA uptake and protein expression by regenerated myocytes following tissue damage. However, in this study, we elucidated a previously unexpected effect of cardiotoxin in generating a favorable immune microenvironment at vaccination sites. When combined with novel fusion vaccines, the combination elicited potent T-cell immunity and antitumor effects. Mechanistically, cardiotoxin induces cytokine and chemokine production, which recruits and activates APC and induces upregulation of signaling molecules by DC for activation of antigen-specific T-cell response. The infiltration of these antigen-presenting DC is critical to the cardiotoxin-associated adjuvant effect, as depleting DC abrogated antigen-specific T cell immunity. Although inflammatory infiltrates were previously described in the cardiotoxin-induced microenvironment, the attention was mostly focused on the role of neutrophils and macrophages in muscular repair and regeneration. Significant increase in NK and CD8⁺ T cells was also described; however, our findings in the DC depletion study indicate that presentation of antigens to infiltrated CD8⁺ T cells directly by DNA-bearing myocytes may not be the principal immune mechanism. Rather, DC recruited during the sterile inflammation was required to present antigens for T cell priming. A similar mechanism has been reported for the adjuvant effects of TLR7 agonists, supporting the role of induction of sterile inflammation as an immune potentiating mechanism.

A large volume of injection relative to the tiny volume of mouse muscle can also cause physical muscle damage and subsequent inflammatory reaction. However, in previous studies, we compared different adjuvants head-to-head for their potential of improving lymphoma idiotype DNA vaccine-induced antitumor effects. These adjuvants included cardiotoxin, crotoxin, MPL, Poly I:C, TLR7/8 agonists. All were given by IM injection using the same volumes, however, only cardiotoxin and crotoxin significantly improved vaccine-induced tumor protection, suggesting the immune potentiating effect is less likely the result of simple injection-associated muscular damage.

An exceptional feature of myotoxin-induced sterile inflammation is the induction of a range of chemokines (Figure 3 and 4) that may have potent chemotactic effects on immature DC, which capture and process expressed antigens for T-cell priming (cross-priming, Figure 6). Alternatively, direct transfection of DC with antigen-encoding plasmid DNA may result in loading antigens into the MHC class I pathway for priming antigen-specific T cells (direct priming, Figure 6). Direct antigen presentation is supported by the observation that cardiotoxin enhanced anti-tumor immunity elicited by a DNA vaccine encoding unfused, non-secreted idiotype antigen (sFv+CT, Figure 1A). Nevertheless, we observed that optimal immunity was elicited by DNA vaccines designed for secretion and loading of DC as exogenous antigens (MCP3-sFv+CT, Figure 1A). We are currently further characterizing the antigen-presentation mechanisms involved.

Another distinctive feature of sterile inflammation induced by myotoxins is activation of innate immunity. TLR expression, inflammatory cytokines, and danger signals upregulated by myotoxin-treated muscular tissue constitute a favorable immune microenvironment for DC maturation and subsequent vaccine-induced adaptive T-cell immunity (Figure 4). For example, it was reported that TLR2 signaling promotes DC activation by certain endogenous ligands, among which heat shock proteins and high mobility groups box 1 protein (Hmgb1) are well-identified. These intracellular molecules are abundantly released from damaged tissues during inflammation and became accessible to DC and activate DC through Myd88-dependent TLR2 pathway. Our observation of up-regulation of TLR2 and Hmgb1, together with significantly reduced antigen-specific T-cell immunity in Myd88 deficient mice, indicates that TLR2 signaling is required (Figure 5C-D). Similarly, CD40 ligation is required for DC maturation. Our results, which showed that antigen-specific T-cell responses were abrogated in CD40-deficient mice (Figure 5A and B), suggests it is also required for the immune potentiating effect associated with myotoxin-induced sterile inflammation. Type I interferons also connect innate to adaptive immunity by its direct effects on activation, expansion and survival of T cells, as well as development of memory T cell immunity. Production of type I interferon by DC is essentially regulated by a number of IRF family members including IRF8, IRF 3 and IRF5, all of which were upregulated in cardiotoxin-treated muscle. Finally, compared with a vaccine adjuvant with a single mechanism of action, such as synthetic reagents that promote DC maturation, exemplified by TLR agonists, a pleiotropic inducer of sterile inflammation may be superior in generating a microenvironment that contains multiple factors stimulating antigen presentation and T-cell priming (Figure 6).

The recommended doses of DNA vaccine used in human study are 1-2 mg. Without any adjuvant, DNA vaccination is not very immunogenic at these doses. For example, a Phase I study showed that vaccinating patients with a lymphoma idiotype DNA vaccine induced idiotype-specific T-cell immunity only in one out of 12 treated patients. These doses are equivalent to 0.25 - 0.5 μ g plasmid DNA in mice. At such low doses, *de novo* protein translation was undetectable upon vaccinating mice with plasmid DNA on cardiotoxin-treated muscles. It is thus required to test the immune adjuvant effect of cardiotoxin on a non-human primate model of that

experimental data will be informative for subsequent human studies. We recently reported antigen-specific T-cell immune response as the result of vaccinating rhesus macaques with a novel HIV DNA vaccine; however, the potency of vaccine-induced immune response was suboptimal without the aid of adjuvants. Future studies to optimize this novel HIV DNA vaccine will include the use of cardiotoxin as an immune adjuvant.

When cardiotoxin is used as an immune adjuvant for prophylactic vaccines against infectious diseases, its adverse effect of myotoxicity is always a safety concern. However, when it is used in combination with cancer vaccines, a greater toxicity profile may be acceptable. A phase I study was conducted to gather the safety and pharmacokinetic information of cardiotoxin in patients with refractory cancers. The results showed that cardiotoxin was well tolerated. With an intensive treatment of 30 consecutive days by IM injection at different doses, local pain at the injection site, reversible diplopia and palpebral ptosis were described as the major toxicities. Similar adverse effects were observed when using crotoxin, an analogue of cardiotoxin, in patients with advanced cancer. The safety profile of these myotoxins in humans supports the clinical potential of cardiotoxin used as an immune adjuvant for cancer vaccines.

In summary, our data showed that cardiotoxin-induced sterile inflammation at vaccination sites generates an innate immune microenvironment that facilitates the induction of potent adaptive immunity in combination with genetic vaccines. Induction of local inflammation may be a promising strategy for breaking immune tolerance or enhancing immunogenicity of infectious pathogen or tumor antigens.

Figure 1

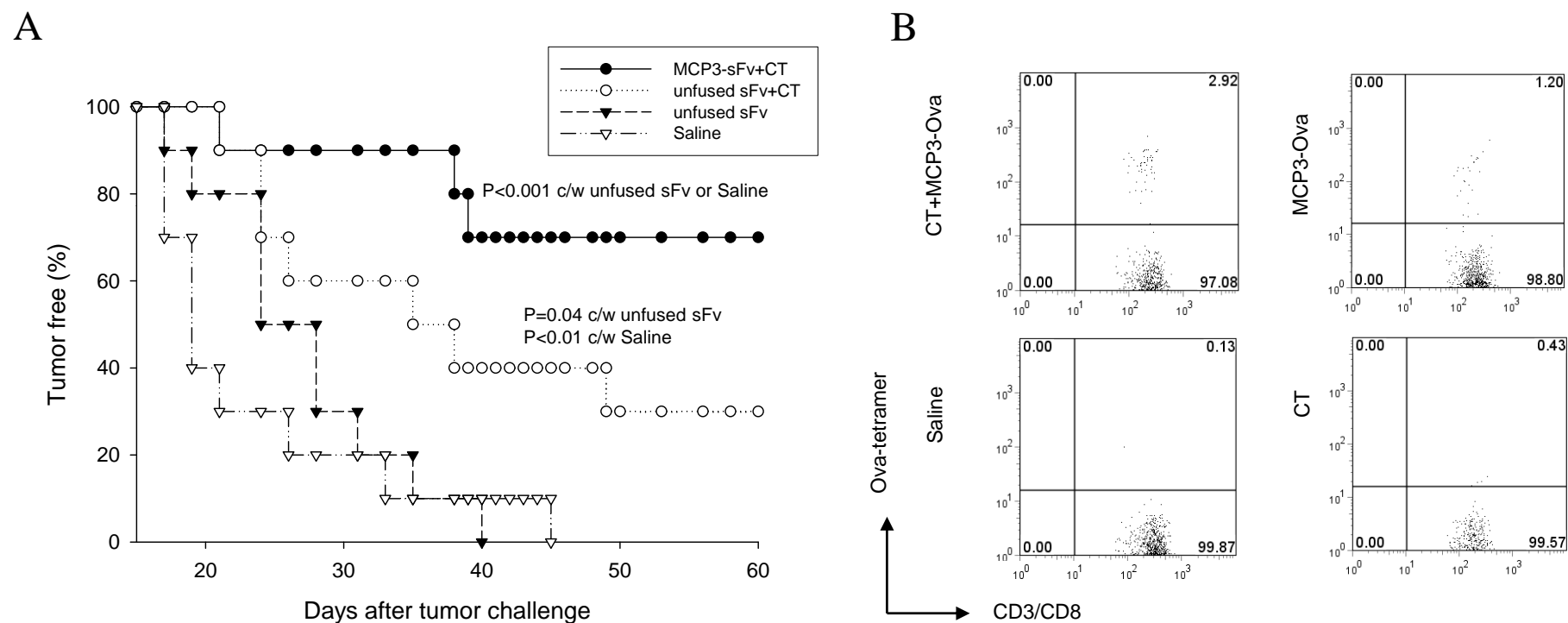


Figure 2

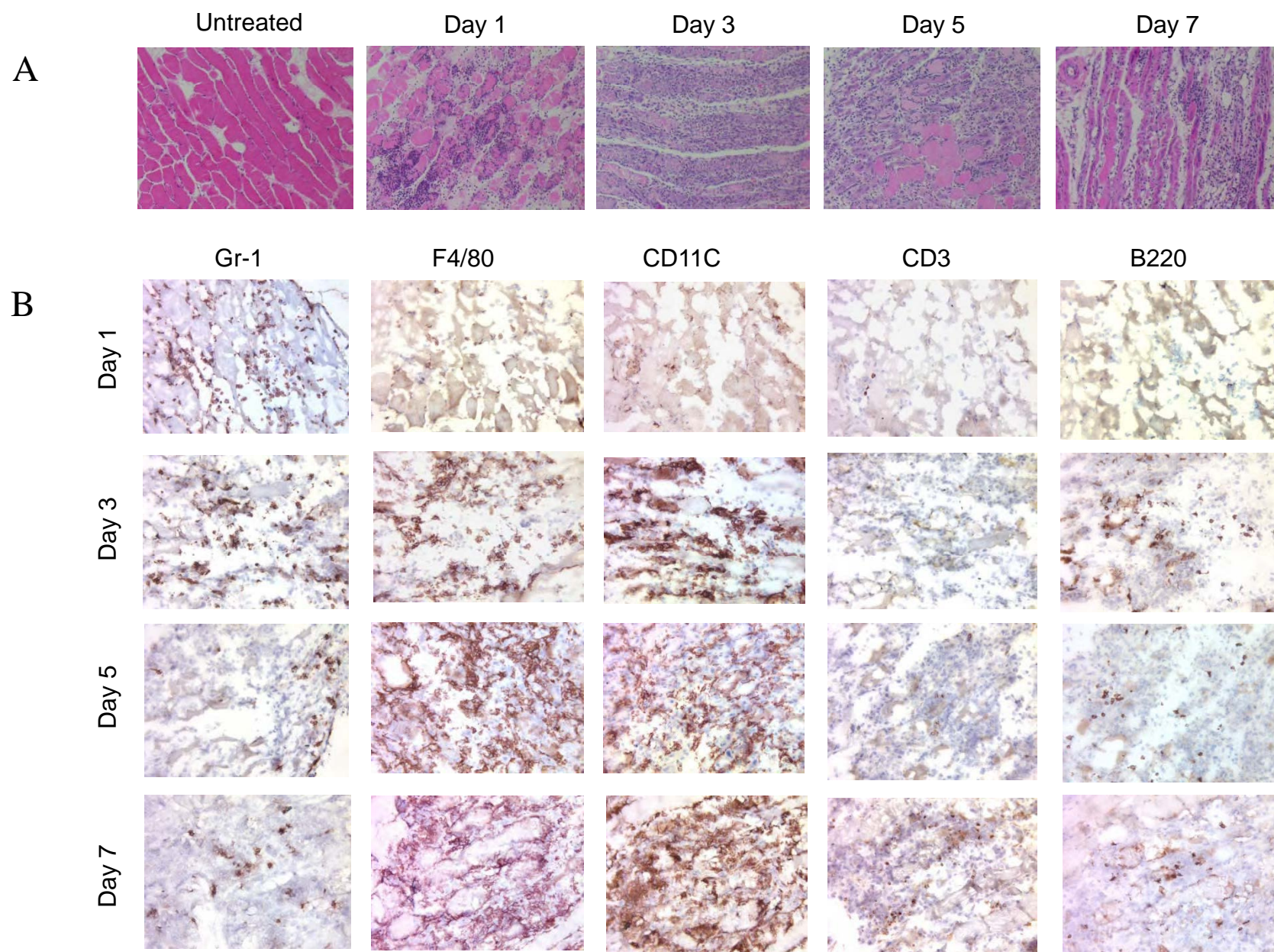


Figure 3

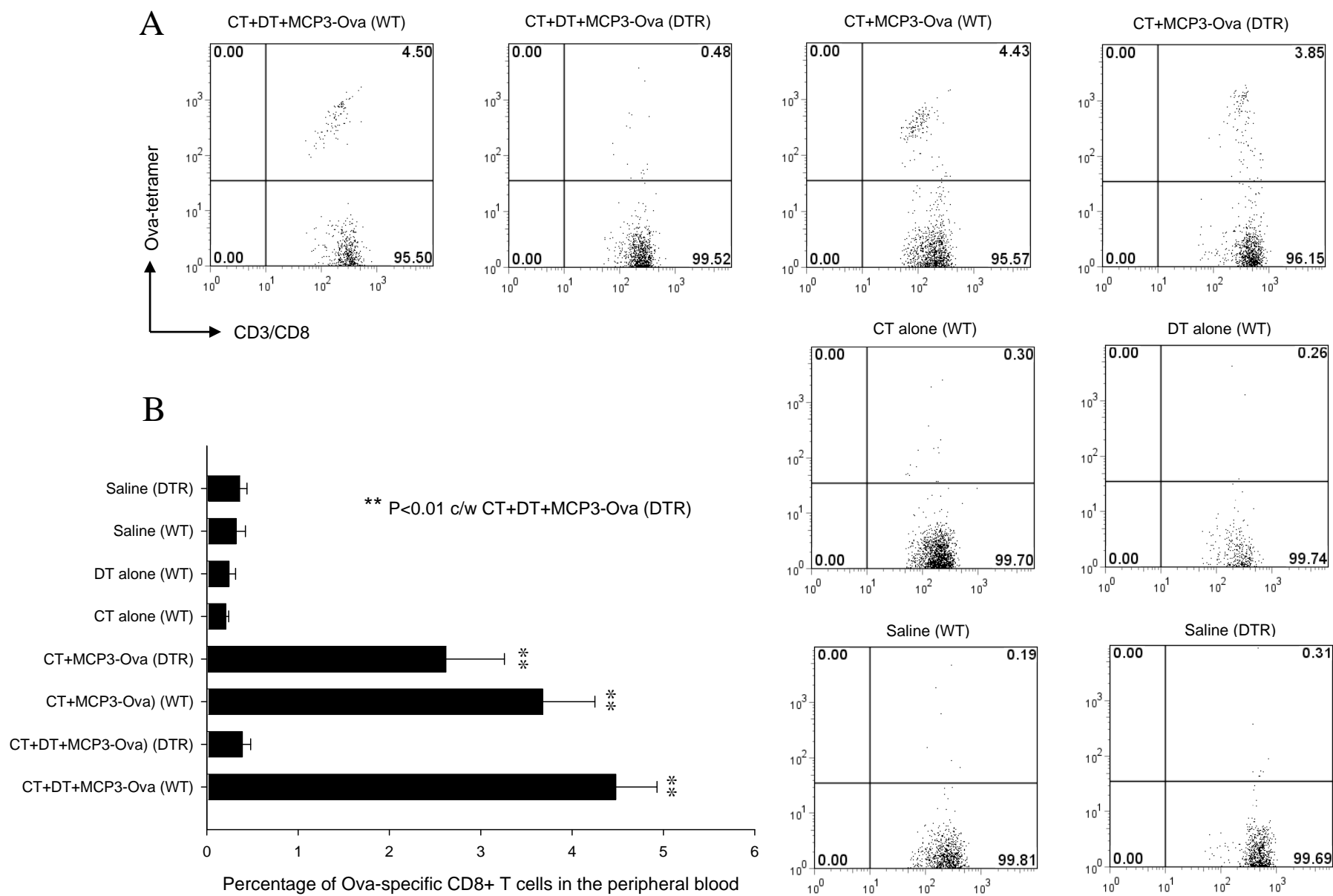


Figure 4

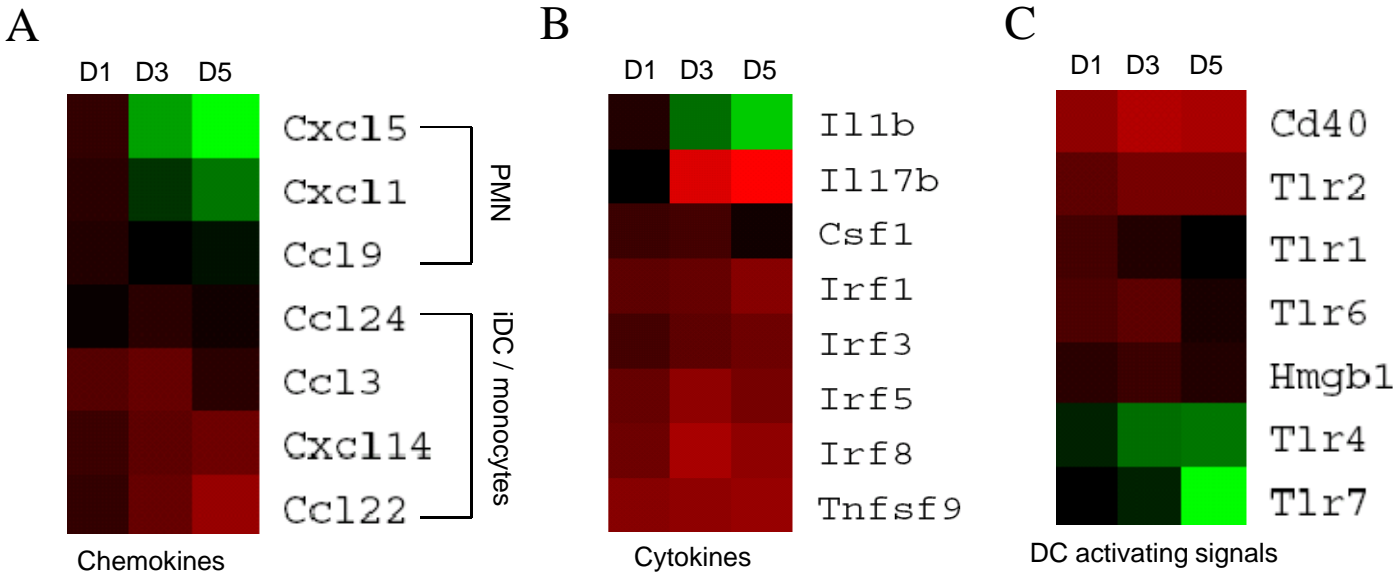


Figure 5

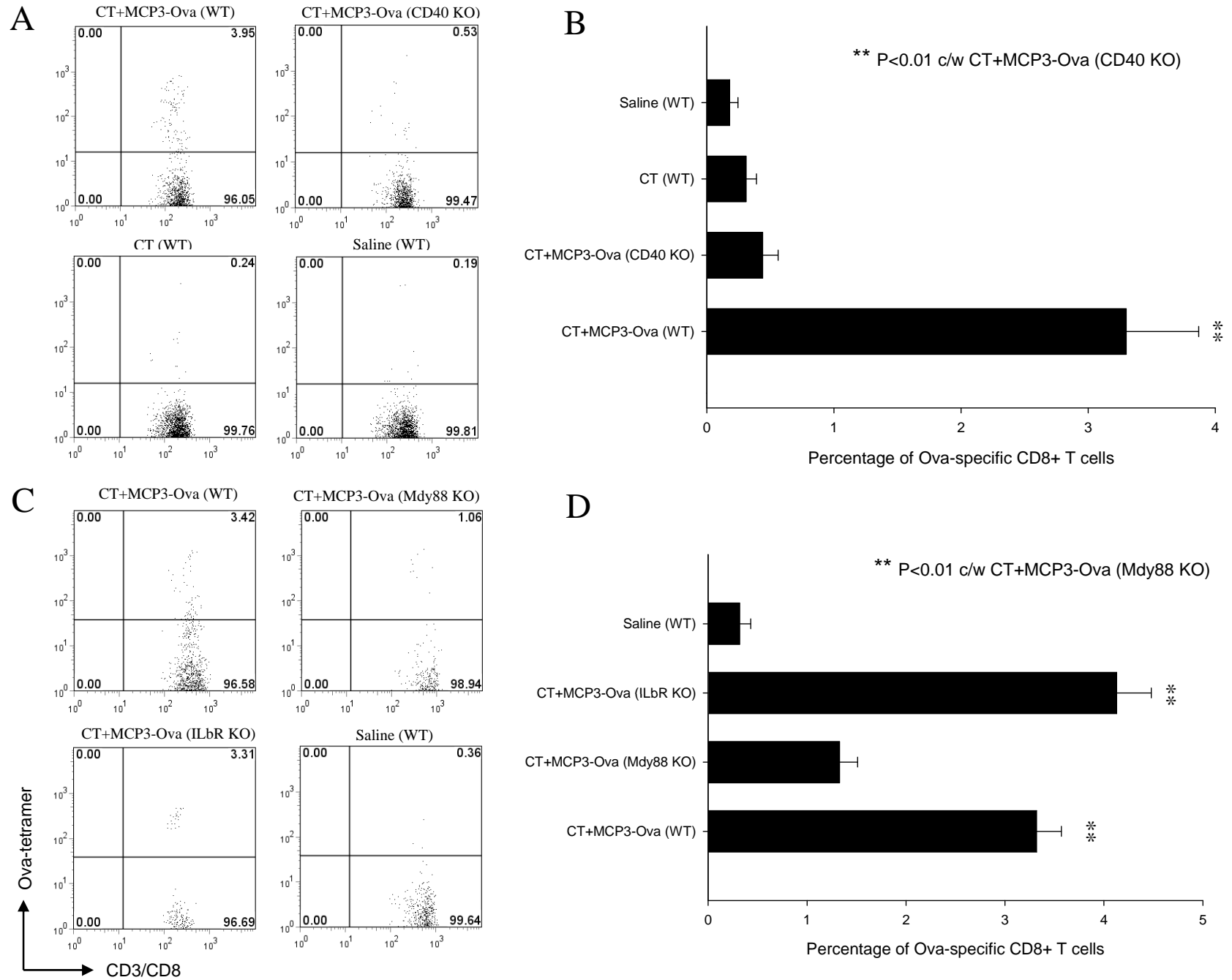


Figure 6

